CHAPTER 6

General Discussion, Conclusions and Future Perspectives
DISCUSSION

MSI profiles change during the adenoma to carcinoma sequence, but not between colorectal and endometrial carcinomas

Microsatellite instability (MSI) is the predominant type of genetic instability present in the tumors of Lynch syndrome patients and also in a subset of sporadic colorectal and endometrial tumors (Boland et al., 1998). It is generally accepted that MSI can be found in the early stages of tumor progression, such as at adenoma level. Studies comparing patterns of MSI in different tumor types and stages suggest that different levels of instability are observed in tumors originating in different tissues or in different stages of tumorigenesis (Furlan et al., 2002; Kuismanen et al., 2002). However, the frequencies reported by different studies vary widely and data on the qualitative differences are scarce.

In chapter 2 of this thesis we analyzed mononucleotide and dinucleotide MSI markers to define specific qualitative MSI profiles in colorectal adenomas and colorectal carcinomas. We included tumors from Lynch syndrome patients and from sporadic cases in order to elucidate possible differences between tumors of hereditary and sporadic origin. In chapter 3 we focused on MSI profiling of colorectal carcinomas versus endometrial carcinomas. We looked at features such as instability frequencies, type of microsatellite mutations, and the size of these mutations in order to evaluate whether these features are tissue-dependent and thus might reveal distinct profiles of MSI between tumors of distinct origin.

We found differences in MSI frequencies during the transition from adenoma to carcinoma, as expected from the literature (Shibata et al., 1994; Grady et al., 1998; Loukola et al., 1999; Iino et al., 2000; Sugai et al., 2003; Giuffrè et al., 2005). The adenomas in our study showed a significantly lower proportion of MSI-H cases than the colorectal carcinomas, both in non-carriers and in truncating mutation carriers. Considering the different distributions of instability, our results from the adenomas were of particular interest. It was mainly the dinucleotide markers that were unstable in colorectal adenomas from non-carriers, whereas a very low frequency of mononucleotide instability was seen, resembling the MSI-L CRC of non-carriers. In contrast, mononucleotide instability was generally predominant in the adenomas of truncating mutation carriers. Based on these data we suggest
that mononucleotide instability is a very early event in the carcinogenic process of tumors with mismatch repair mutations, and that mononucleotide instability precedes that of dinucleotide repeats in such tumors. We further hypothesize that the dinucleotide instability in non-carriers represents a kind of background instability, as seen in MSI-L CRC tumors, which is not a sign of an underlying MMR deficiency, whereas in Lynch syndrome tumors, with proven MMR deficiency, mononucleotide instability can be considered to truly result from the underlying MMR deficiency.

A possible explanation for these findings might be that the normal MMR system more easily corrects mismatches in mononucleotides than in dinucleotides. We assume that if more instability is seen in dinucleotide repeats in MMR-proficient tumors (which are less frequent repeats in the genome than mononucleotide repeats) then this suggests either a higher vulnerability of dinucleotide repeats to the occurrence of mismatches and/or a lower capacity of the normal MMR system to repair them.

In chapter 3 we suggest that it is not possible to define clearly different MSI profiles distinguishing MSI-H CRC and EC, as we observed that the MSI-related features that we studied showed similar patterns in both types of carcinomas. The frequency of mononucleotide and dinucleotide instability found in both types of tissues is comparable, with mononucleotide and dinucleotide markers being affected at similar levels. In terms of type of microsatellite mutation, mononucleotide markers virtually always become shorter, whereas dinucleotide markers can become shorter and/or longer, both in CRC and EC. This close association of the occurrence of insertions or deletions with the type of MSI marker suggests that characteristics of the repeats, such as repeat length, have a bigger influence on the type of mutation occurring at a microsatellite repeat than the tissue origin of the tumor in which those mutations arise. Differences between CRC and EC could be seen in terms of the size of the deletion/insertions detected, with EC having shorter alterations than CRC. However, the relative differences between the markers remained similar in both tissue types, leading to comparable patterns of instability. We propose that the differences observed might indicate different durations of tumor development and/or differences in tissue turnover between
Implications for diagnostics

Since we observed that the MSI-H adenomas from mutation carriers mainly showed mononucleotide instability, our results have implications for the diagnosis of Lynch syndrome colorectal adenomas. Our results indicated that analyses of mononucleotide markers are advantageous for identifying colorectal adenomas and carcinomas associated with Lynch syndrome. To our knowledge, our data are the first to show that the use of a panel of only mononucleotide markers, as previously recommended for the detection of MSI-H hereditary CRC (Buhard et al., 2004), can also be used to advantage in the identification of Lynch syndrome patients by testing colon adenomas.

With respect to the MSI detection in carcinomas, we suggest that the same MSI tests can be used for both colorectal and endometrial tumors, as we found no great differences between the EC and CRC MSI profiles; the differences were not enough to justify using different MSI tests for the two tumor types.

Identification of new target genes for MSI tumors: genes in the estrogen-receptor pathway are good candidates

Genes containing repeats are frequent targets of mutations in MMR-deficient tumors. Particularly mutations accumulating at coding sequences of important regulatory genes (target genes) have been implicated in the development of MSI tumors. The profile of target genes affected in MSI-H CRC has been well established, with several genes being highly mutated, such as TGFβ-RII which has a mutation frequency of approximately 90% in these tumors. For MSI-H EC, the profile is less well known, or at least genes having such a high mutation frequency have not been identified so far. Previous studies suggest that the profile of target genes differs between endometrial and colorectal carcinomas and that frequently mutated target genes remain to be found in the EC (Duval et al., 2002).

In chapter 4 of this thesis we described our work on the identification of new target genes for EC. We identified 44 genes that were mutated in the MSI-H EC we examined, of which seven were mutated relatively frequently. We propose these
seven genes – NRIP1, SRPR, MBD6, JAK1, KIAA1009, JMJD1C, and ADD3 – as new target genes for MSI-H EC. They encode proteins with several functions, with some already reported to play a role in cancer. Interestingly, the most frequently mutated gene in EC in our study was NRIP1 (34%). This gene encodes a co-repressor protein of the estrogen-receptor (ER) pathway. The ER is an essential pathway for endometrial tissue regulation; the endometrium is a hormonal-responsive tissue, highly regulated by the concentrations of estrogens. Several estrogen-responsive genes have already been described, and genetic alterations in ER and those ER-responsive genes are thought to be key players in the development of hormone-associated tumors, such as endometrial carcinomas (Notarnicola et al., 2001). High exposure to estrogens is currently considered the major risk factor for developing EC. Approximately 80% of all sporadic EC tumors – the endometrioid endometrial carcinomas – are estrogen-associated carcinomas (Doll et al., 2008). NRIP1 has been described as essential for female fertility in mice (White et al., 2000), and mutations in NRIP1 may act as a predisposing factor for human endometriosis (Caballero et al., 2005). We believe that it is very likely that the NRIP1 mutations we found in our MSI-H endometrial carcinomas might result in functional differences at the ER-pathway level. We expect inactivation of NRIP1 to interfere with the process of co-repression of the ER complex and lead to differences in the expression of estrogen-responsive genes that could eventually result in tumor growth advantages, as previously observed in breast cancer studies (White et al., 2005).

We further showed that most of these genes are also mutated in colorectal and gastric tumors, although in different frequencies. These results confirm that some target genes show tissue specificity, while others seem to play a more common role in MSI-H tumors, independently of the tissue origin. We were surprised to find NRIP1 mutations in colorectal carcinomas. All the reasons mentioned above make NRIP1 an obvious target gene for EC, but a less obvious one for CRC. However, this gene has already been reported as a target gene for gastrointestinal MSI tumors, despite their lower mutation frequencies in such tumors. Frameshift mutations were found in an A9 coding microsatellite, in 13% of MSI-H GCs and in 7% of MSI-H CRC (Røyrvik et al., 2007). Furthermore, although not a typical hormone-associated cancer, CRC does have a hormone component.
The presence of estrogen receptors and products of estrogen-related genes in the colon suggests that estrogens have a role in the organization and architectural maintenance of the colon, and their down-regulation accompanies the progression of CRC (Francesca et al., 2008). Moreover, some studies suggest that the combined estrogen and progesterone hormone replacement therapy might be the factor underlying the reduction of incidence of CRC in postmenopausal women (Chlebowski et al., 2004). In addition, CRC incidence and mortality rates are lower in females than in males. Some authors have therefore suggested that estrogens have a protective effect against CRC (Wada-Hiraike et al., 2006). Slattery et al. (2001) explored the contribution of several estrogen-related factors to the differences in MSI tumor frequency observed in men and women, and also in younger versus older women. They showed that withdrawal of estrogen may increase the risk of MSI-positive CRC. In fact, our finding of NRIP1 mutations in the CRC group reinforces the possible link between this particular gene (and also the ER pathway) and MSI carcinogenesis.

This subject is more thoroughly addressed in chapter 5, in which we tried to clarify the mechanisms linking hormones to Lynch syndrome-associated tumors and, in particular, to discuss how hormones could play a role in MSI tumorigenesis. Our data suggest that there might be a stronger link between hormones and MSI than so far thought, and that genes of hormone-related pathways, such as the ER pathway, might be good candidates for target genes in MSI-H tumors, not only for estrogen-responsive tissues, such as the endometrium, but also for other tissues such as the colon.

CONCLUSIONS

Our data have provided new insights into the process of MSI-H related tumor development. Our results suggest that mononucleotide instability is a very early event in the carcinogenic process of colorectal tumors with mismatch repair mutations, and that mononucleotide instability precedes that of dinucleotide repeats in such tumors. We therefore propose that analyses of mononucleotide markers are advantageous for identifying colorectal adenomas and carcinomas.
associated with Lynch syndrome. Furthermore, we showed that there were no substantial differences of MSI profile between CRC and EC, thus we propose that the same MSI tests can be used for both colorectal and endometrial tumors. We also found mutations in coding microsatellite repeats likely to indirectly affect the estrogen-receptor pathway in MSI-H tumors. Our data suggest that genes in the ER pathway would be very good candidate genes for mutation analysis in MSI-H, and possibly also in microsatellite-stable tumors. These findings could prove interesting in the design of novel therapeutic treatments.

**FUTURE PERSPECTIVES**

Although the work presented in this thesis gave many new insights in MSI and in the development of MMR-related tumors, it also raised many questions and it opened avenues for further research in this field.

With respect to the new findings on microsatellite instability patterns of colorectal adenomas and carcinomas, it would be very interesting to conduct further (basic) experiments. It would for instance be of great interest to obtain a large set of micro-dissected adenoma and carcinoma tissues from the colon of the same Lynch syndrome patient to evaluate at a larger scale the differences in MSI profile between adenomas and carcinomas. Moreover, analyzing several micro-dissected samples of different areas of the same MMR-deficient adenoma would give us great insight in this matter. Finding only mononucleotide instability or simultaneous mono- and dinucleotide instability, and no areas with only dinucleotide instability, would corroborate our hypothesis that mononucleotide repeats are targeted first in adenomas with MMR mutations. Finding this in colorectal adenomas raises the question whether this holds true for all MMR-related cancers. Extending such studies to other types of Lynch syndrome cancers, such as endometrial or gastric, would answer these questions.

The same applies to our project comparing MSI profiles in CRC and EC. Although we are convinced that for these two tumor types the patterns of MSI are comparable, we do not know whether the same holds true for all MMR-related tumor types. Therefore, it would be good to compare colorectal tumors with all
other MMR-related tumors. Without these data we do not know whether the MSI test generally used is good for all MMR-related tumor types.

Considering our project on target genes, studies at the functional level will be of major relevance to show how the genes identified in this project are implicated in tumor development and progression. Silencing of these genes \textit{in vitro} and \textit{in vivo}, and analyzing the effects on tumor-associated processes, such as proliferation, apoptosis or invasion, would be of great help to elucidate the effects of the identified truncating mutations in those genes on cancer development. Studying the highest mutated gene, \textit{NRIP1} gene, could not only prove the involvement of this gene in cancer development, but also it could help us in understanding the complicated field of hormone-responsive pathways and their involvement in mismatch repair- deficient tumors. Finding one member of a pathway mutated might indicate that other members of the same pathway might also play a role in tumor development; in case of \textit{NRIP1}, other members of the ER pathway, a pathway frequently targeted in therapeutic procedures for estrogen-responsive cancers, seem to be appealing targets to study in MMR-deficient tumors. Their possible involvement and knowledge of their mechanism of action might lead to a better understanding of tumor response or tumor resistance to some hormone-related therapies.
REFERENCES


105


